EXHIBIT D

AXON DEGENERATION MECHANISMS: COMMONALITY AMID DIVERSITY

Michael Coleman

Abstract | A wide range of insults can trigger axon degeneration, and axons respond with diverse morphology, topology and speed. However, recent genetic, immunochemical, morphological and pharmacological investigations point to convergent degeneration mechanisms. The principal convergence points — poor axonal transport, mitochondrial dysfunction and an increase in intra-axonal calcium — have been identified by rescuing axons with the slow Wallerian degeneration gene (*Wld*^{S}) and studies with blockers of sodium or calcium influx. By understanding how the pathways fit together, we can combine our knowledge of mechanisms, and potentially also treatment strategies, from different axonal disorders.

WALLERIAN DEGENERATION
The degeneration of an axon distal to a site of injury, which begins to occur about 1.5 days after a lesion.

AMYLOID PRECURSOR PROTEIN
(APP). A membrane glycoprotein component of fast axonal transport, from which Aβ is cleaved by proteolytic processing.

SODIUM–CALCIUM EXCHANGER
The protein that couples sodium influx to calcium efflux. This may function in reverse if intracellular sodium concentrations increase.

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Recent studies show that axon degeneration precedes, and sometimes causes, neuronal death in several disorders[1–5], so the need to understand its mechanisms is compelling. This is a challenging task because axon degeneration is directly triggered by a diverse range of insults, including injury, toxins and genetic defects, and is a common secondary event in inflammation, metabolic disturbances, myelin disorders and ischaemia[6,7].

Our understanding of other cell death pathways, such as apoptosis and autophagy, has typically begun with predictions based on morphology, which were later confirmed with genetic, immunochemical and pharmacological tools[8,9]. Morphological studies of axon degeneration have shown various degrees of swelling, apparent differences in topology, axonal transection (in some disorders but not others) and different speeds of degeneration (see examples below). These differences have sometimes been interpreted as evidence for multiple mechanisms, but it is important to remember that our understanding of the morphology of axon degeneration is incomplete, largely because of the macroscopic dimensions involved.

A series of important developments has now given us the genetic and pharmacological tools to block axon degeneration in specific circumstances, and new immunochemical and imaging methods to determine the sequence of biochemical and cellular changes. The slow Wallerian degeneration mutant (*Wld*^{S}) mouse, in which WALLERIAN DEGENERATION is delayed for 2–3 weeks, has been used to show that both physical injury and a blockade of axonal transport trigger a proactive axon death programme, the molecular details of which are just beginning to emerge[10]. Immunocytochemistry for AMYLOID PRECURSOR PROTEIN (APP), a marker for impaired axonal transport, has revealed similar patterns of axon damage in a wide range of disorders[6]. Transgenic mice that express analogues of green fluorescent protein (GFP) in neuronal subsets have revolutionized longitudinal and live axon imaging[11]. Finally, pharmacological blockade of sodium channels and the SODIUM–CALCIUM EXCHANGER has revealed a pathway of axon degeneration common to several disorders and provided the most immediate prospects for therapeutic intervention[12].

The emerging information points to convergent pathways and demands a rethink of axon degeneration mechanisms and their classification. This review assesses the current data concerning how and where the mechanisms converge, and highlights future challenges and opportunities in the field.

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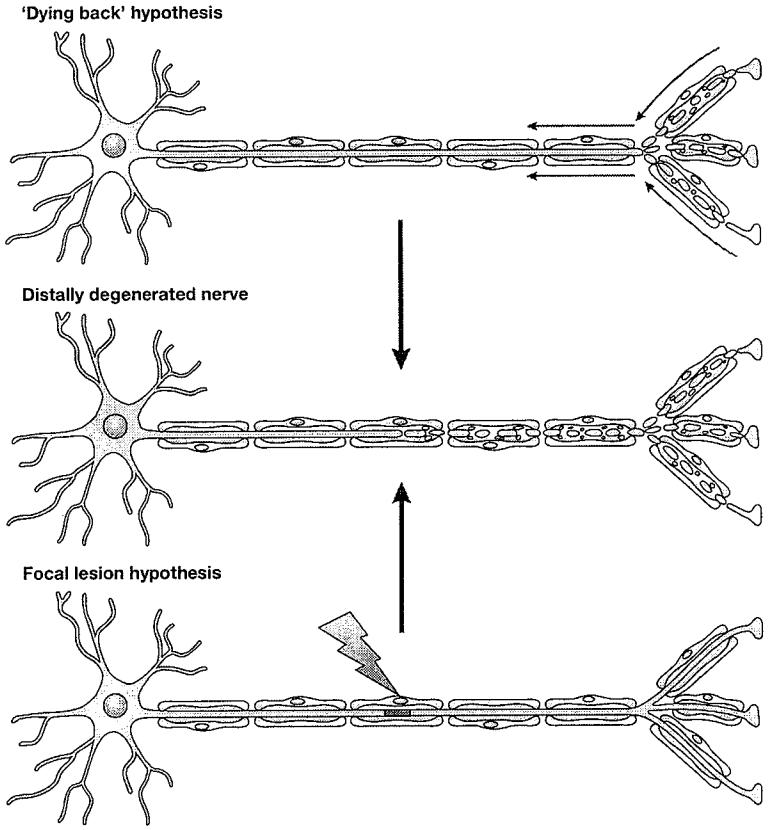

Figure 1 | **Dying back and focal lesion models of axon degeneration.** Shows two alternative models to account for the observation that nerves in many disorders show greater axon degeneration at their distal ends (centre). The 'dying back' model (top) proposes that degeneration of each axon starts at the distal end and moves retrogradely. The focal lesion model (bottom) proposes that focal lesions can trigger Wallerian degeneration of distal axons, while proximal axons remain intact. The lesion does not necessarily need to transect the axon, as a focal block of axonal transport (red bar) might also trigger Wallerian degeneration.

CHARCOT-MARIE-TOOTH DISEASE
Hereditary neuronopathy that involves both sensory and motor axons. Can originate from defects in myelin (type I) or axons (type II).

TAXOL
A microtubule-stabilizing drug used in cancer chemotherapy. Because of its action on microtubules, side effects of Taxol include peripheral neuropathy. Taxol dosage must be limited for this reason.

AXONAL SPHEROID
Focal swelling of an axon, usually in the CNS, to many times its usual diameter, typically swelling to 10–50 μm. Spheroids are often filled with disorganized cytoskeleton and organelles, and many stain positively for APP.

Wallerian degeneration in injury and disease

Wallerian degeneration is a simple experimental model of axon degeneration, in which the distal stump of an injured axon degenerates rapidly after a reproducible latent phase[13–15]. There has been a long-standing debate about the relationship between Wallerian degeneration and the large group of toxic and genetic disorders known as 'dying back' neuropathies, in which axon degeneration is most prominent in distal nerves. Similar dying back processes may underlie the early loss of synapses that occurs in Alzheimer's disease[16].

Axon degeneration in dying back disorders seems to be indistinguishable from Wallerian degeneration when studied at a single site[17,18], but apparent differences in the directionality of degeneration have caused confusion. To make matters worse, this directionality has been controversial in both fields, and there is early loss of neuromuscular synapses in Wallerian degeneration that does not necessarily mirror the behaviour of the rest of the axon[19,20]. The dying back hypothesis holds that degeneration begins at the distal ends of diseased axons and spreads retrogradely[17] (FIG. 1). Reports that injury-induced Wallerian degeneration spreads anterogradely[14,21], although controversial (see below), raised doubts as to whether dying back disorders and Wallerian degeneration could be related. However, an alternative hypothesis for the mechanism of dying back degeneration is more clearly linked to Wallerian degeneration. According to this hypothesis, one or more focal lesions, not necessarily transecting the axon, trigger degeneration of the whole axon distal to their sites[18,22]. In adult animals, the proximal axon often remains intact, which offers an explanation for why nerves show more axon degeneration at their distal ends without needing to infer a retrograde spread of degeneration.

With the discovery of the *Wld*^{S} mouse[23], the hypothesis that Wallerian degeneration and dying back are related pathways could be tested. In *Wld*^{S} mice, injury-induced Wallerian degeneration is delayed ~tenfold (for 2–3 weeks) by a dominant mutation that acts intrinsically in neurons[24–26]. In crosses with progressive motor neuronopathy (*pmn*) mice[1] and myelin protein zero (P0) null mutants — a model of CHARCOT-MARIE-TOOTH DISEASE[27] — *Wld*^{S} significantly delayed dying back axon degeneration, thereby proving the mechanistic link to Wallerian degeneration. Similar findings in TAXOL toxicity studies suggested that toxic disorders also trigger a Wallerian-related pathway[28]. Symptoms in all three disorders were significantly delayed in *Wld*^{S} mice. These key studies confirmed Augustus Waller's bold prediction that Wallerian degeneration is important "particularly with reference to nervous diseases"[13] a century and a half after it was proposed.

In the CNS, *Wld*^{S} also protects against both genetic and toxic insults, as well as transient global cerebral ischaemia[29]. Some nigrostriatal axons, which degenerate in Parkinson's disease, are spared and remain functional after 6-hydroxydopamine lesions in *Wld*^{S} mice[30], and AXONAL SPHEROIDS are reduced in number in the GRACILE TRACT of mice with gracile axonal dystrophy (*gad*)[31], which lack ubiquitin carboxyterminal hydrolase L1 (UCHL1)[32]. The significance of this is that axonal spheroids, or smaller VARICOSITIES, which can be broadly termed AXONAL DYSTROPHY, are almost universal in neurodegenerative diseases of the CNS, probably as manifestations of a major pathway of CNS axonal death. The similarity to Wallerian degeneration was unexpected, because Wallerian degeneration — at least in the PNS — involves only slight axonal swelling[15,33]. However, new morphological studies suggest that swelling is also prominent in CNS axons during Wallerian degeneration[34] (see below). These results raise the possibility that axonal swelling in many CNS disorders reflects a Wallerian-related mechanism (defined as one that shares at least one step with Wallerian degeneration). Axonal swelling disorders include traumatic brain injury[35], Alzheimer's disease[36], Parkinson's disease[37], Creutzfeldt–Jakob disease[38], HIV dementia[39] and multiple sclerosis[40,41].

ACUTE AXON DEGENERATION (AAD) of both the proximal and distal stumps of transected spinal cord axons has also recently been linked to Wallerian degeneration.

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Table 1 | **Effectiveness of *Wld*^{S} in blocking axon degeneration**

Insult	Nature of insult	Age of onset or acute lesion	Effectiveness of *Wld*^{S}
Nerve transection	Physical	Acute	2–3 week delay[15,23]
Nerve crush	Physical	Acute	2–3 week delay[15,23]
Taxol	Toxic	Acute	>2 week delay[28]
6-hydroxydopamine	Toxic	Acute	Some axons preserved for at least 11 days[30]
pmn	Genetic	3 weeks	Strong protection: 2–3 week delay[1]
P0 null	Genetic	6 weeks	Effective at 3 months, but not at 5.5 months[27]
gad	Genetic	6 weeks	Reduces pathology at 4 months, but no improvement in symptoms[31]
SOD1 G93A transgene	Genetic	11 weeks	No axon protection, modest extension of lifespan[42]
SOD1 G37R transgene	Genetic	4–5 months	No protection at 5–6 months[43]
SOD1 G85R transgene	Genetic	9–10 months	No protection at ~1 year[13]
Plp null	Genetic	8–18 months	No protection at 18 months[41]

gad, gracile axonal dystrophy; *Plp*, proteolipid protein; *pmn*, progressive motor neuronopathy; P0, myelin protein zero; *SOD1*, superoxide dismutase 1.

AAD is delayed by *Wld*^{S}, which shows that it shares some regulatory features with Wallerian degeneration[34]. AAD might be important for attempts to repair spinal cord lesions, because dying back of the proximal stump allows more time for a glial scar to form before any regenerating axons reach the lesion site, and can lead to the loss of other axon branches.

Not all axon degeneration is delayed by *Wld*^{S}. Exceptions include the superoxide dismutase 1 (*SOD1*) transgenic mouse models of amyotrophic lateral sclerosis (ALS)[42,43], the proteolipid protein (*Plp*) null model of hereditary spastic paraplegia[44] and neurite degeneration caused by botulinum neurotoxin C1 *in vitro*[45]. *Wld*^{S} is effective *in vitro* against a range of other insults[25,46–48]. The failure to protect axons in some circumstances could indicate the existence of other axon degeneration pathways. Alternatively, the trend for *Wld*^{S} to be more effective in early-onset or acute disorders (TABLE 1) might reflect the fact that a delay of 2–3 weeks is easier to detect in more rapidly degenerating axons. The protective effect of *Wld*^{S} for synapses (but not axons) also declines with age[20], so *Wld*^{S} might not prevent symptoms in older animals even when it does protect axons[27,31]. Interestingly, *Wld*^{S} seems to protect synapses more robustly in rats, which suggests that its potential usefulness might not be fully reflected in mouse models of human disorders[49].

The significance of these studies is threefold. First, the *Wld*^{S} gene is a valuable experimental tool with which to manipulate axon degeneration and determine its role in disease. Second, *Wld*^{S}, or its putative downstream axonal mediators, once they are identified (see below), might be of therapeutic use in some disorders. The mechanism by which this is achieved depends on the nature of the pathway. Third, Wallerian degeneration has been validated as an experimental model that can be used to study axon degeneration mechanisms in some human disorders. In many ways it is more suited to experimental study than the disease itself. The investigator has control over the exact timing, site and nature of the lesion, so the ensuing events can be more accurately defined.

GRACILE TRACT
An axon tract in the dorsal spinal cord that carries proprioceptive axon branches from the dorsal root ganglion to the medulla oblongata.

VARICOSITIES
Minor swellings of axons, typically of less than 10 μm.

AXONAL DYSTROPHY
Generic term for misshapen axons in pathology that encompasses both larger spheroids and smaller varicosities.

ACUTE AXON DEGENERATION (AAD). Rapid retraction of proximal and distal axon stumps from a site of spinal cord transection, which occurs ~30 min after the lesion.

POSITIONAL CLONING
Identification of a gene on the basis of its chromosomal location.

SILENT INFORMATION REGULATOR
(Also known as sirtuin). NAD-dependent deacetylase that is involved in gene silencing and can influence longevity in some species.

The *Wld*^{S} gene

The *Wld*^{S} gene, which was identified using POSITIONAL CLONING[10,50], encodes an in-frame fusion protein of the amino (N)-terminal 70 amino acids (N70) fragment of ubiquitination factor E4B (UBE4B), the complete coding region of NAD^+ synthesizing enzyme nicotinamide adenylyltransferase 1 (NMNAT1) and a unique 18-amino acid linking region. Because of the unusual *Wld*^{S} mutation[51], the wild-type proteins continue to be expressed normally[20,50]. Surprisingly, WLD^S protein seems to be confined to the nucleus *in vivo*[10,27,30,52], which suggests the existence of downstream axonal mediators, although the possibility that WLD^S acts at very low levels in axons has not been ruled out, and would fit with some *in vitro* observations[53]. Identifying other mediators of this pathway is now a key aim in the field.

At present, which parts of the WLD^S sequence are required for axon protection and which downstream factors are involved are controversial issues. NMNAT1 has been reported to be sufficient for axon protection *in vitro*, acting either through the SILENT INFORMATION REGULATOR (or sirtuin, SIRT1)[54] or through local NAD^+ synthesis in neurites[53]. However, these results are inconsistent with observations made *in vivo*, because transgenic mice that overexpress NMNAT1 show a normal rate of Wallerian degeneration[7] (L. Conforti and M. C., unpublished observations), and because WLD^S protein is not present at a detectable level in

axons *in vivo*[10,27,30,52]. This suggests that NMNAT1 and WLD^S are not interchangeable, although whether NAD^+ synthesis is necessary for axon protection *in vivo* remains to be resolved. The requirement for the UBE4B-derived N70 sequence for axon preservation *in vivo* might underlie the delay in axon degeneration by proteasome inhibition[55,56]. However, contrary to an earlier proposal[57], the ubiquitin–proteasome system seems to function normally in *Wld*^{S} axons[52]. The direct effects of WLD^S seem to be subtle and confined to the nucleus[58].

There are several candidates for the events that mediate the WLD^S regulatory pathway, but no consistent theme has yet emerged. In the nucleus, the N70 domain of WLD^S binds and redistributes the key ubiquitin–proteasome system component VALOSIN-CONTAINING PROTEIN (VCP, also known as p97) (H. Laser, L. Conforti, M. C. *et al.*, unpublished observations), and pituitary tumour transforming gene 1 (PTTG1) expression is altered in *Wld*^{S} mice[59]. In neurites in primary culture, extracellular-signal regulated kinase 1 or 2 (ERK1/2) phosphorylation is required to mediate a delay in Wallerian degeneration by proteasome inhibitors[56], and RhoA (a small GTPase) activation accelerates Wallerian degeneration, although RhoA might not be activated in degenerating peripheral nerves[60]. A trypsin-like serine protease activity is also required for normal Wallerian degeneration *in vitro*, acting before the depletion of ATP[61] (see below). *In vivo*, a Schwann cell response to axonal injury can be detected through activation of the receptor tyrosine kinase ERBB2 minutes after axotomy[62]. Although events in Schwann cells are unlikely to influence the intrinsic axonal decision to degenerate[24–26], this is evidence of a rapid axonal signalling pathway, another branch of which could trigger programmed axonal death. Future work could show whether one or more of these events mediates, or is blocked by, the action of WLD^S, but it will be important to demonstrate these effects *in vivo* as well as *in vitro*.

Transport failure triggers axon degeneration

The most obvious common feature of models in which *Wld*^{S} delays axon degeneration is a blockade of axonal transport from the cell body. Normal microtubule functioning, which is essential for axonal transport, is prevented by mutation of the tubulin-specific chaperone e (TBCE) gene in *pmn* mice[63] and by the microtubule-stabilizing action of Taxol. Dysmyelination in P0 null mice may constrict axons to impair transport[64], and accumulation of APP is evidence of defective axonal transport in the axons of *gad* mice, although how this is connected to the UCHL1 mutation is unknown[65]. Nerve transection is the ultimate block of axonal transport from the cell body, and, interestingly, when axonal transport is severely blocked in *pmn* mice[63], *Wld*^{S} causes a 2–3 week delay in axon degeneration that is strikingly similar to that observed after transection. Wallerian-like degeneration also results when axonal transport is disrupted in other ways, such as by colchicine treatment or mutation of neurofilament proteins[66,67]. The fact that a Wallerian-related mechanism can be triggered without physical axonal injury rules out calcium influx at the transection site as the initiating event. An interruption of axonal transport from the cell body seems to be a likely alternative trigger for Wallerian degeneration.

Axonal transport is a complex bidirectional process that involves many motor proteins[68]. Genetic defects in kinesin family proteins often specifically cause axon degeneration[69,70], whereas defects in retrograde transport also cause cell body death[71], which suggests that anterograde transport might be particularly important for preventing axon degeneration. However, Wallerian degeneration is no longer considered to be an atrophic process caused by failure to deliver structural components of the axon[14]. The atrophy eventually shown by *Wld*^{S} axons differs both morphologically and temporally from Wallerian degeneration in wild-type mice[15], so a much faster mechanism seems to account for Wallerian degeneration. The delay in Wallerian degeneration by genetic mutation and the fact that it can be triggered by a wide range of insults suggest instead a regulated, proactive death programme akin to apoptosis[46,72]. Factors that execute this programme must be constantly present in the axon, but maintained in an inactive form before transection, just as carefully regulated effectors of apoptosis are always present in cells. The role of anterograde axonal transport could be to deliver a putative natural inhibitor of Wallerian degeneration.

At the molecular level, no link between Wallerian degeneration and apoptotic pathways has been established. The *Wld*^{S} gene reveals no similarity to anti-apoptotic factors[10], and mutations that block apoptosis, such as B-cell leukaemia/lymphoma 2 (BCL2) overexpression or BCL2-associated protein X (BAX) and BCL2 agonist killer 1 (BAK) deletion, do not prevent axon degeneration in injury or disease[73–75]. Activation of the apoptotic effector caspase 3 and products of caspase-mediated degradation are not detectable in injured axons, and caspase inhibition does not block Wallerian degeneration[76]. Conversely, *Wld*^{S} does not block apoptotic death of the cell body[23]. As there seem to be no molecular clues from apoptosis, the identity of the *Wld*^{S} gene will be important in helping to elucidate the mechanism of Wallerian degeneration.

Transport failure in the CNS

The results described above suggest that blockage of anterograde axonal transport might trigger axon degeneration. In parallel with these findings, impairment of axonal transport has been found in many CNS neurodegenerative disorders. APP, which is normally transported through axons by fast axonal transport without reaching detectable levels, can accumulate in axonal spheroids or varicosities, providing an immunocytochemical marker for blockages of axonal transport (FIG. 2). APP accumulation was first found in axons that had been damaged by traumatic brain injury[77], then in multiple sclerosis[40] and subsequently in a host of other disorders (BOX 1). Therefore, like the

VALOSIN-CONTAINING PROTEIN
An essential component of the ubiquitin–proteasome system that transfers multi-ubiquitinated proteins to the proteasome in endoplasmic reticulum, and has other known and unknown cellular roles.

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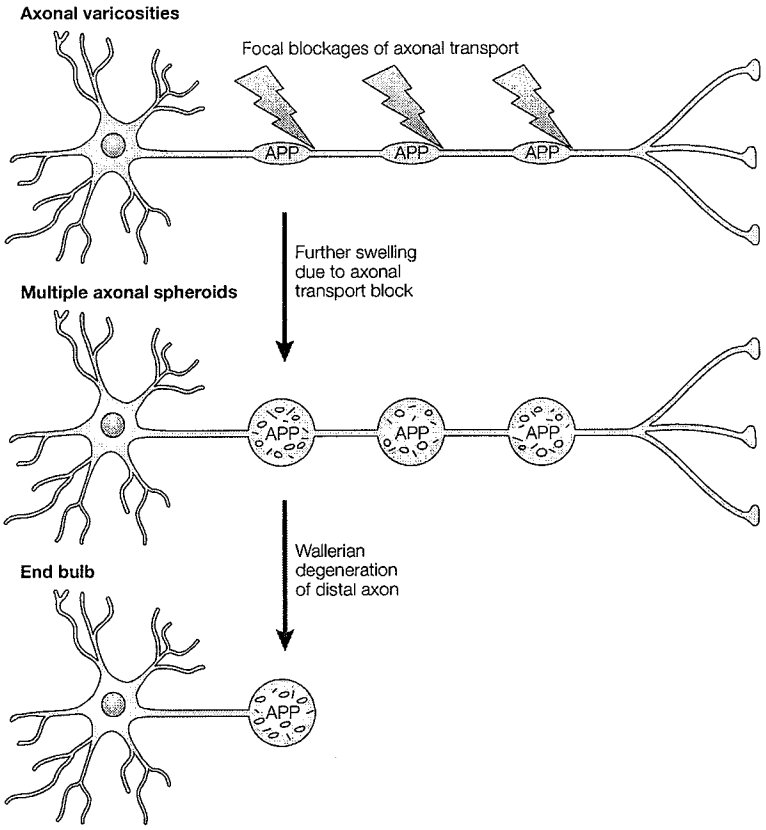

Figure 2 | **Axonal varicosities, spheroids and end bulbs.** One proposed sequence of events in the development of axonal spheroids and end bulbs. Focal blockages of axonal transport, which may occur preferentially at nodes of Ranvier[44], lead to accumulation of organelles and disorganized cytoskeleton in axonal varicosities (top). Amyloid precursor protein (APP) also accumulates in these. The swellings increase in size to form axonal spheroids (centre). Up to this point the axon remains continuous[3,36,79–81], but as the spheroids grow, axonal transport may become increasingly impaired. Eventually, the block of axonal transport is of sufficient magnitude to trigger Wallerian degeneration of the distal axon (bottom). An end bulb remains on the proximal axon stump. End bulbs also form when axons are transected directly, so transection cannot be ruled out as a cause of their appearance in disorders such as multiple sclerosis. However, in this case, the observed varicosities and spheroids on continuous axons would also have to be explained.

Wld^S gene, APP immunocytochemistry is changing the way we look at axon pathology, and indicates that the mechanisms are more closely related than previously thought. In combination, the model in which impaired anterograde axonal transport triggers Wallerian degeneration and the frequent blockage of axonal transport in disease raise the possibility that Wallerian-like mechanisms account for axon death in many CNS disorders.

In some CNS disorders in which APP accumulates, impaired axonal transport is not just a consequence of axon damage, but part of the cause. In *gad* mice, the ability of *Wld*^{S} to delay axon pathology[31] suggests the existence of a pathway that shares steps with those involved in *pmn* and Taxol toxicity. In many disorders, APP accumulates in moderately swollen axons[6,40], and in traumatic brain injury it accumulates before disruption of the axolemma[78], which suggests that axonal transport failure is an early event. In models of Alzheimer's disease, deliberate impairment of axonal transport increases not only APP accumulation, but also amyloid deposition and plaque formation, raising the intriguing possibility that amyloid-β (Aβ) is produced at sites of APP accumulation[3]. Poor axonal transport has not yet been firmly established as a causal event in these disorders, but these reports are consistent with such a role. The specific roles of anterograde and retrograde axonal transport in disease also need to be better understood. A wider perspective comes from considering whether axonal swelling is an early or late event in axon degeneration.

Axonal spheroids form early in pathogenesis

It is essential to know whether axonal spheroids arise as terminal END BULBS after axons degenerate, or whether their growth precedes loss of axonal continuity (FIG. 2). The importance of this question is twofold: to understand the sequence of events that lead to axon degeneration, and to know whether axon swelling is a potentially reversible stage of pathogenesis, or simply a manifestation of terminal axon damage. In multiple sclerosis, the prevailing view is that axons become transected by poorly understood inflammatory events and the severed ends then swell to form end bulbs[40,41]. However, there is little direct evidence for a transection event *in vivo*, and observations of spheroids and varicosities on continuous axons in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE)[41,79] suggest a different sequence of events, in which axon swelling precedes Wallerian degeneration of distal axons.

New morphological methods have revealed that spheroids arise on continuous axons in a wide range of disorders. Longitudinal axon imaging, which is necessary to address this question, is intrinsically difficult, especially in the CNS. Axons have macroscopic dimensions and are bundled together in thousands, so images must be simplified to follow individual axons over any significant distance. This is now possible using transgenic mice that express spectral variants of GFP in representative neuronal subsets[11]. In the YFP-H (yellow fluorescent protein) and GFP-S mouse lines, for example, INSERTIONAL SILENCING of the transgene in most neurons means that nervous system images are simplified by about 50-fold, so that single axons can be traced over several centimetres in peripheral nerves and hundreds of microns in the CNS[15,33,34,80].

The resulting longitudinal images in models of Alzheimer's disease[36], ALS[80] and gracile axonal dystrophy (R. Adalbert and M. C., unpublished observations), which are also supported by conventional imaging[3,79,81], clearly show that spheroids and varicosities begin on unbroken axons in many CNS disorders. Moreover, spheroids frequently appear not singly, but as tandemly-repeated swellings on individual axons (FIG. 2), which suggests a multifocal block of axonal transport not unlike the focal lesion model of dying back disorders (FIG. 1). The striking similarities between diverse disorders again suggest similar mechanisms.

END BULBS
Large swellings of up to 50 μm in diameter that develop terminally on both proximal and distal axon stumps after transection.

INSERTIONAL SILENCING
Silencing of transgene expression by genomic elements at the site of integration.

Box 1 | **CNS disorders with APP-positive axon pathology**

Acute demyelinating disorders[118] | Alzheimer's disease[3] | Creutzfeldt–Jakob disease[38] | Gracile axonal dystrophy[65] | HIV dementia[119] | Human T-cell lymphotropic virus 1 (HTLV1)-associated myelopathy[6] | Malaria[6] | Multiple sclerosis[40] | Parkinsonism-dementia of Guam[120] | Spinal cord injury[6] | Stroke[121] | Traumatic brain injury[77] | White matter ischaemia[122]

A failure of axonal transport would be most likely to cause axons to swell if it were localized, causing a bottleneck. The recurrent involvement of nodes of Ranvier suggests that axonal transport through nodes may be particularly vulnerable. In *Plp* null mice, poor retrograde transport causes organelle accumulation at the distal PARANODE, which results in axonal swelling[44,82]. Intriguingly, similar observations were made many years ago in peripheral nerves 24 h after crush injury, although swelling is less extensive in peripheral nerves[83–85]. Mice deficient in 2′,3′-cyclic nucleotide phosphodiesterase (CNP1) show numerous axonal swellings despite the presence of normal myelin[86]. CNP1 is required for paranodal integrity, and for clustering nodal sodium channels and paranodal adhesion proteins such as contactin-associated protein (CASPR)[87]. Importantly, dispersal of the sodium channel Nav1.6 is also a feature of APP-positive axons in multiple sclerosis and EAE[88,89]. In the PNS, one of the few disorders showing significant axon swelling — giant axonal neuropathy — also begins at the paranode[22].

Therefore, a working model for axonal spheroid pathology involves localized failure of axonal transport, particularly at nodes of Ranvier, which causes excessive build up of axoplasm, including rapidly transported proteins such as APP. Failure of axonal transport from cell bodies eventually triggers Wallerian degeneration of distal axons, leaving proximal axon stumps with large end bulbs.

PARANODE
Region adjacent to nodes of Ranvier, where a series of cytoplasmic loops form septate-like junctions with the axon, acting as a diffusion barrier between node and internode.

AMYLOID PLAQUES
Sites of Aβ accumulation and dystrophic neurites in the brains of mouse models and patients with Alzheimer's disease.

Aβ IMMUNOTHERAPY
Amyloid clearance from the brains of mouse models of Alzheimer's disease using antibodies to Aβ.

CALPAINS
Ubiquitous calcium-dependent cysteine proteases that are strongly implicated in the later stages of Wallerian degeneration.

BEPRIDIL
A broad-range calcium channel blocker with a strong inhibitory effect on sodium–calcium exchange and on sodium channels.

New horizons in axon imaging

The fact that axonal swellings arise on continuous axons raises the question of their reversibility. This question is best addressed by live axon imaging, a technique made possible by the endogenous and harmless nature of the label in YFP-H mice. Live imaging can be applied both to tissue explants to study the effect of varying culture conditions[33,90] and *in vivo* to study truly physiological events. Transcranial two-photon imaging has shown axonal spheroids developing from normal axons around AMYLOID PLAQUES in Alzheimer's disease models[36], and apparently regressing after Aβ IMMUNOTHERAPY[91]. Although more data are needed to firmly establish spheroid reversibility, the prospect is an exciting one, and demonstrates the new horizons being opened up by these imaging methods.

Other longstanding questions about spatiotemporal patterns of axon degeneration are also being addressed. The progressive nature of Wallerian degeneration might hold valuable clues to its mechanism. This has been a controversial matter for decades, with conflicting reports that axons degenerate from the cell body in a proximal to distal manner[14,21], a distal to proximal manner[92], or simultaneously along their lengths[93]. These earlier studies were limited to statistical analyses of axons at several discontinuous points along the nerve because long-range longitudinal imaging was not feasible. As Wallerian degeneration is highly asynchronous in the axon population[14,15,34], it is clearly preferable to follow the behaviour of individual axons along the length of the nerve.

Using the transgenic mouse lines YFP-H and GFP-S[11], Wallerian degeneration has now been imaged along 3 cm lengths of individual PNS axons[33] and over hundreds of microns in the CNS[34]. By targeting the short time window when most axons are degenerating, one of the key predictions of a progressive model has been confirmed: that axons pass through a stage during which they degenerate at one end but not at the other[15,34]. However, this stage is short-lived. In sciatic and tibial nerves, only ~5% of axons are partially degenerated at any one time, the rest being either fully intact or fully degenerated. The lifetime of the transition state may be less than an hour and the speed of progression seems to be faster than that of fast axonal transport[15,34], which challenges earlier suggestions that clearance of factors by continued anterograde axonal transport in the distal stump triggers degeneration[21].

The length of the latency phase of Wallerian degeneration in these experiments (a little over 35 h[15,34]) and the catastrophically rapid degeneration that follows it are strikingly similar between the CNS and PNS branches of young mouse dorsal root ganglion (DRG) axons. The proximal to distal direction of progression after axon transection is also consistent between these two environments, although there is an unexplained reversal of direction in crushed sciatic nerves[15]. These data, together with the ability of *Wld*^{S} to block Wallerian degeneration in both the CNS and PNS, suggest that the respective mechanisms are similar.

Pharmacological rescue of axons

Pharmacological studies also indicate that several disorders share a final pathway of axon degeneration. An influx of extracellular calcium is necessary and sufficient to induce Wallerian degeneration, probably triggering a final stage of cytoskeletal degradation by activated CALPAINS[94,95]. During the long latent phase of Wallerian degeneration, when morphological and ultrastructural changes are limited, a steady but significant increase in intra-axonal calcium parallels a prolonged sodium leak into the axon[96], which, in turn, might result from depletion of ATP[47,53]. A causal link between rising axonal calcium and the increase in sodium concentration was established by blocking sodium channels with tetrodotoxin, which also prevented the calcium increase[96]. Reverse operation of the sodium–calcium exchanger seems to mediate this link, as the calcium increase is also blocked by BEPRIDIL. A similar mechanism seems to operate in axonal stretch injury, a model of traumatic brain injury[97].

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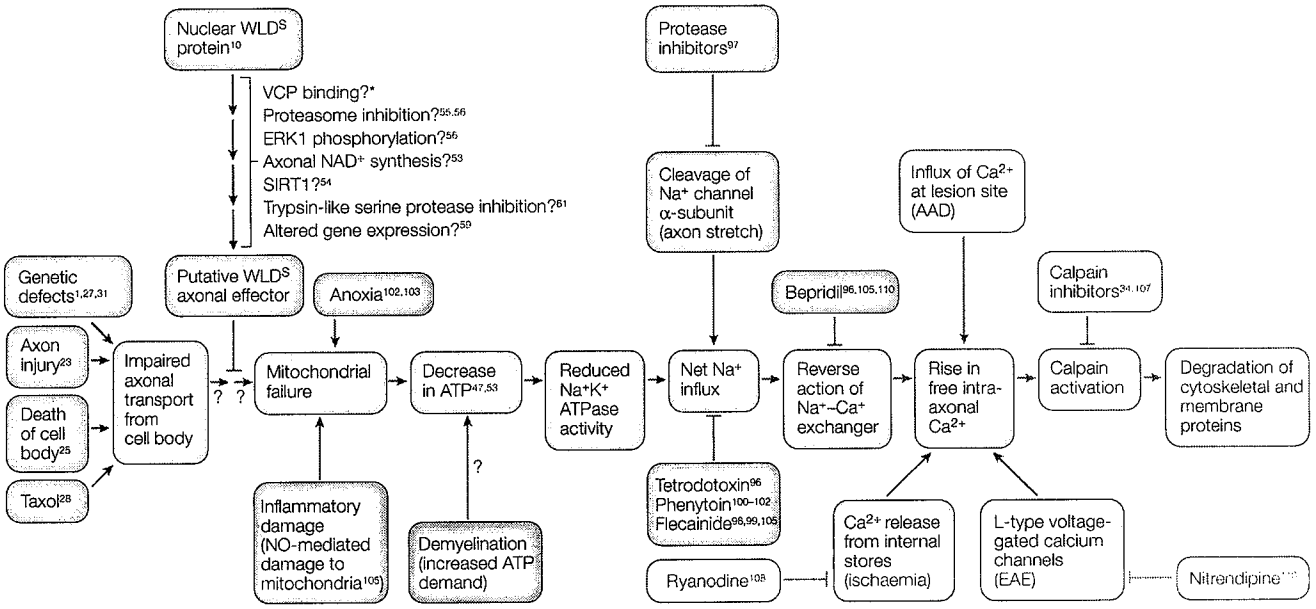

Figure 3 | **Convergent pathways of axon degeneration.** Known mechanisms of axon degeneration appear to channel through to calpain-mediated degradation of axonal proteins. Upstream of this, there are three main convergence points: impaired axonal transport from the cell body, mitochondrial failure and an increase in intra-axonal calcium. AAD, acute axon degeneration; EAE, experimental autoimmune encephalomyelitis; ERK1, extracellular-signal regulated kinase 1; NO, nitric oxide; SIRT1, silent information regulator; VCP, valosin-containing protein; WLD^S, slow Wallerian degeneration protein. *Data from H. Laser *et al.*, unpublished observations.

These findings suggest a promising therapeutic approach to axon degeneration. Blocking sodium channels with PHENYTOIN or FLECAINIDE, or blocking the sodium–calcium exchanger with bepridil, alleviates symptoms in animal models of progressive and chronic relapsing EAE, experimental autoimmune neuritis (EAN), anoxia and spinal cord contusion[98–103], although the effect of these drugs on inflammatory cells remains to be clarified[104]. Sodium channel blockade also preserves axons exposed to nitric oxide[105]. Some of these treatments are now moving towards clinical trials for multiple sclerosis[106]. Calpain inhibition is also effective in an animal model of Taxol toxicity, which offers further support for a calcium-based mechanism of axon damage[107].

Other aspects of calcium metabolism might also influence axon degeneration. In ischaemic injury, part of the increase in free intra-axonal calcium is attributable to release from intracellular stores, as blocking this release protects axons[108]. Intra-axonal calcium is also redistributed below SCHMIDT-LANTERMAN INCISURES within 4 h of axon injury[109], long before any detectable increase in total calcium concentration[96], which might also reflect release from internal stores. L-type voltage-gated calcium channels also mediate some calcium influx in some disorders, as nitrendipine, a selective calcium blocker, is protective in EAE[110]. Finally, calcium enters transiently through a site of axon transection, probably causing the rapid axon retraction from a lesion site in AAD, which is completely abolished by calpain inhibition[34].

Another treatment that blocks axon degeneration in several models is erythropoietin. Symptoms in diabetic neuropathy and acrylamide-induced peripheral neuropathy and axon degeneration in an *in vitro* model of HIV sensory neuropathy are reported to be alleviated by erythropoietin[111–113], which again suggests an underlying similarity in degenerative mechanisms. Erythropoietin is released by Schwann cells in response to axon injury, an event that might reduce axon degeneration *in vitro*[113]. Although erythropoietin has been reported to block neuronal apoptosis[114] its protective mechanism for axons remains unknown.

Convergent mechanisms of axon degeneration

The genetic, immunochemical, morphological and pharmacological data discussed above all suggest that mechanisms of axon degeneration are more closely related than previously thought, but is there only one mechanism or are there several? This question is best addressed by working backwards from later events to earlier ones.

Several pathways seem to raise the concentration of free intra-axonal calcium, causing calpain activation and degradation of axonal proteins (FIG. 3). As calcium also influences Wallerian degeneration[95], there are no obvious exceptions to these last steps. In many cases, reverse action of the sodium–calcium exchanger causes axonal calcium to rise, but release from intra-axonal stores in ischaemia[108] and influx through the lesion site in AAD also contribute to increases in calcium. The rise in intra-axonal sodium that drives reverse sodium–calcium exchange probably results from an ATP deficit,

PHENYTOIN
A drug that blocks sodium channels and inhibits persistent sodium currents, commonly used as an anticonvulsant.

FLECAINIDE
A sodium channel blocker used extensively as an anti-arrhythmic drug to correct irregular heartbeats.

SCHMIDT-LANTERMAN INCISURES
Regions of non-compacted myelin in peripheral nerves that allow communication between the Schwann cell cytoplasm adjacent to the axon and that on the external surface of the myelin sheath.

MITOCHONDRIAL UNCOUPLING
Prevents the oxidative phosphorylation of ADP to ATP in mitochondria without affecting electron transport, so that respiration no longer yields energy in the form of ATP.

but might be exacerbated by proteolytic cleavage of the sodium channel α-subunit in some disorders[97]. ATP deficiency, in turn, can have many causes and seems to be a key convergence point. For example, nitric oxide may damage mitochondria in inflammatory disorders[105], demyelination raises the energy demand of conduction and anoxia causes a failure of oxidative respiration[103].

Evidence for a further important convergence point is that WLD^S acts upstream of ATP deficiency but can block degeneration in several disorders. ATP is deficient in axons undergoing Wallerian degeneration[47,53], which is consistent with observations of mitochondrial swelling[10,49] and loss of mitochondrial membrane potential[47,115]. These events are all delayed in *Wld*^{S} axons, but MITOCHONDRIAL UNCOUPLING using carbonyl cyanide *m*-chlorophenylhydrazone bypasses the protective effect of WLD^S (REF. 47). Therefore, the putative axonal mediator of WLD^S acts upstream of mitochondrial impairment. A good candidate for the earlier convergence point is a block of anterograde axonal transport, but how this is linked to mitochondrial impairment remains unclear. One clue is that a specific trypsin protease inhibitor can prevent ATP depletion[61] but how this is linked to axonal transport, and whether such an event mediates protection in *Wld*^{S} axons, is unknown.

Conclusions and future directions

Axon death in many disorders follows a proactive, non-apoptotic death programme, which can be triggered in many ways. New experimental tools show that mechanisms are convergent, with poor anterograde axonal transport, mitochondrial dysfunction and an increase in axonal calcium concentration being the principal convergence points. Apparent differences in morphology, topology and speed of axon degeneration do not necessarily reflect fundamentally different mechanisms.

Many key challenges remain. The downstream nuclear and axonal effectors of WLD^S remain unknown. The link between poor axonal transport and ATP deficiency in Wallerian degeneration needs to be understood. It is essential to determine whether axonal spheroids contribute to axon degeneration or are simply manifestations of blocked axonal transport, whether and how they can be reversed, and the role of nodes of Ranvier in their development. Promising preclinical results with blockers of sodium channels and sodium–calcium exchange need to be translated into safe and effective therapeutics in man, and the range of disorders in which they block axon degeneration needs to be determined.

There are also exciting opportunities. First, the potential for combining new tools to study axon degeneration mechanisms has been demonstrated[13,34] but not exhausted. Second, the availability of mice with fluorescently labelled Schwann cells and astrocytes[116] will help us to understand how these cells interact with labelled degenerating axons. Third, the generation of transgenic calcium reporter mice offers the prospect of understanding calcium dynamics in degenerating axons *in vivo*[117].

The convergent nature of mechanisms of axon degeneration offers important opportunities. The time has come to think across the old boundaries and, where appropriate, to combine our knowledge of axon degeneration mechanisms in one disorder with that from another. Equally important, individual therapeutic strategies might protect axons in several disorders. The axon degeneration field can now be compared to apoptosis in the early 1990s. A framework pathway is in place (FIG. 3), which may grow and branch as more mechanisms are studied and new experimental tools developed. By understanding this pathway, some of today's experimental tools may become tomorrow's therapeutic leads.

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Competing interests statement

The author declares no competing financial interests.

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